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Fibroblasts produce brain-derived neurotrophic factor and induce mesenchymal transition of oral tumor cells

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SUMMARY

Fibroblasts (Fibs) contribution to neoplastic progression, tumor growth, angiogenesis, and metastasis has been recently reported by several research groups. In this study it was investigated if fibroblasts are the source of brain-derived neurotrophic factor (BDNF), which plays a crucial role in the progression of oral squamous cell carcinoma.

In a novel *in vitro* system oral Fibs were cultured with SCC-25 lingual squamous cell carcinoma cells for 7 days. Factors related with this interaction were investigated by quantitative PCR and western blot.

In the co-culture, fibroblasts were converted to carcinoma-associated fibroblasts (CAFs), which in return initiated epithelial–mesenchymal transition (EMT) of SCC-25 cells. The induced CAFs produced increased levels of BDNF, which interacted with the increased-expressed neurotrophin receptor B (TrkB) on EMT-converted SCC-25 cells. Possible regulatory factors of BDNF expression (tumor necrosis factor- α and interleukin-1- β) were detected both in CAFs and EMT-tumor cells. In CAFs: IL-1 β -, in SCC-25 cells TNF- α -gene-expression was significantly increased in co-culture conditions.

Activated fibroblasts (CAFs) and mesenchymal transitioned tumor cells might use the BDNF-TrkB axis and its regulation to harmonize their interaction in the process of tumor progression.

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Introduction

Fibroblasts and myofibroblasts often represent the majority of the stromal cells within various types of human carcinomas, yet the specific contributions of these cells to tumor growth are under intensive investigation. Previous studies revealed that, mutual paracrine effects between tumor cells and stroma (myo)fibroblasts lead to tumor cell proliferation and progression.¹ An activated mesenchymal cell population, named carcinoma-associated fibroblasts (CAFs), have been extracted from a number of invasive human carcinomas, which are competent to promote the growth of carcinoma cells.² A functional property of CAFs is the sustained expression of stromal derived factor 1 (SDF-1),³ which plays a central role in the local invasion of cancer.⁴ While the potential importance of CAFs in tumor progression is becoming clear, the generation mechanisms of them from normal fibroblasts, or mesenchymal stem cells are currently under extensive investigation.

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Recently Mishra et al. described an experimental system where CAFs were induced from mesenchymal stem cells by treatment with carcinoma cells-derived medium.³ Dynamic interaction systems between carcinoma and mesenchymal cells are required to understand the interaction between CAFs and tumor cells. It is extremely important to use human cells in these interaction systems, since especially fibroblasts are different in mice in relationship to cancer⁵ and to senescence.⁶

Accordingly, in the current study we describe a novel human *in vitro* tumor–stroma interaction system, which is able to induce CAFs from normal periodontal ligament (PDL) fibroblasts within 7 days.

In tumor cells, stroma microenvironment induces an epithelial–mesenchymal transition (EMT), which is considered as a major biological process in epithelial tumor invasion, progression and metastasis. During this process loss of epithelial cell polarity and morphology is observed together with induction of a mesenchymal phenotype.^{7,8}

Interestingly, very recent studies provided evidence that neurotrophin receptor B (TrkB), a 145-kDa receptor tyrosine kinase and its ligand: brain-derived neurotrophic factor (BDNF) may be co-opted in the regulation of EMT in head and neck squamous cell carcinoma (HNSCC).^{9,10} In addition, altered TrkB expression, signaling

and mutations have been found to be important in various other cancer types, including carcinomas of the pancreas, lung, colon and prostate, as well as neuroblastoma and multiple myeloma.¹¹

Hypothesis

In this study we hypothesized that, the main ligand of TrkB: BDNF is produced by CAFs, and the BDNF-TrkB axis is a regulatory way in harmonization of induction of CAFs in the stroma and induction of EMT in the tumor cells. For testing this hypothesis an in vitro experimental system co-culturing periodontal ligament fibroblasts with SCC-25 lingual squamous cell carcinoma cell line was developed.

Materials and methods

Cell lines

Periodontal ligament (PDL) fibroblasts were received from Prof. Dr. Miosge (Department of Prosthodontics, Georg-August-University, Göttingen, Germany).¹² PDL fibroblasts were routinely cultured in DMEM-low glucose (PAA, Linz, Austria) supplemented with 10% fetal bovine serum (FBS) (PAA), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin. SCC-25 cells were purchased from the German Collection of Mikroorganismen and cell cultures (Braunschweig, Germany), and were routinely cultured in DMEM/F12 (PAA, Linz, Austria) supplemented with 10% FBS (PAA), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin.

Co-culture

For induction of carcinoma-associated fibroblasts (CAFs), a modified protocol of transwell cultures was used, based on a previous report,¹³ and detailed described in the [Supplementary material 1](#).

RNA extraction, reverse transcription and PCR

Total RNA was isolated from control and co-cultured cells as described before.¹⁴ Reverse transcription was completed by utilization of the “iScript cDNA Synthesis Kit” (Bio-Rad, Munich, Germany). Real time quantitative PCR (qPCR) was performed using the Quantance Sensimix Sybr & Fluorescein Kit (Foster City, CA, USA) in a MyiQ cycloer (Bio-Rad).

PCR primers for β-actin,¹⁵ E-cadherin, vimentin, snail¹⁶ and SDF¹⁷ were described before. β-actin functioned well as house-keeping gene, and did not show significant changes under experimental conditions in fibroblasts and in SCC-25 cells.

Other primers (BDNF, TrkB, TNF-alpha, IL1β) were designed by using the program Primer Blast of NCBI (NIH, Bethesda, MD, USA) primers sequences are summarized in [Supplementary material 2](#). The relative gene expression was calculated as reported before.¹⁴

Protein fractionation, immunoprecipitation and western blot

Cells after co-culture and controls were scraped into 500 µl extraction buffer/well or/insert, non-nuclear and nuclear protein fractionation was performed as described previously.¹⁸ Non-nuclear protein fractions were subjected to protein concentration measurement,¹⁸ and 16 µg proteins were used for immunoprecipitation with Protein A-magnetic beads (Roth, Karlsruhe, Germany), and 1 µg TrkB-reactive-rabbit immunoglobulin (Biovision, Mountain View, CA, USA), following the instructions of the manufacturer. Controls were done by immunoprecipitation using 1 µg TrkB-reactive-

rabbit immunoglobulin preincubated for 1 h with 1 µg blocking peptide. Protein extracts prepared in the same way, containing 16 µg proteins from TrkB-overexpressing SH-SY5Y cells were used as positive controls.¹⁹ Precipitated proteins were removed from beads into sample buffer: 250 mM Tris HCl pH = 6.8, 4% SDS, 10% glycerol, 0.006% bromphenolblue, by 5 min incubation at 70 °C, followed by western blotting using a previously published protocol,²⁰ and a rabbit polyclonal antibody of Santa Cruz Biotechnology, (Santa Cruz, CA, USA).²⁰ The first immunoprecipitation supernatants were subjected to western blot of β-actin (Sigma, Vienna, Austria) for control of equal loading.²¹ Density of the detected bands was done by the Image J software.¹⁴

Statistical analysis

Each experiment was performed in two independent sets containing at least three biological repeats/set, altogether 7 repeats were performed. Each biological repeat was done in two technical repeats. The relative gene-expression results were tested for normal distribution by D’Agostino & Pearson omnibus normality test using the Graphpad Prism 4.03 (Graphpad Software Inc.). Significance of changes in co-culture vs. controls was tested by one-way analysis of variance within each set using SPSS 15.0 Software. The independent experimental sets were then compared for reproducibility. Only reproducible significant changes were considered as “significant”. Significance was declared by the standard $p < 0.05$ level. Correlation analysis was performed to determine the relation of gene expression changes by SPSS.

Results

TrkB in human oral carcinoma

Using a polyclonal rabbit antibody, TrkB immunohistochemical labeling was recognised in several tissue samples of head and neck squamous cell carcinoma. As already described by Smit et al.,⁹ the TrkB reactivity was recognised in tumor cells of HNSCC, and an increased reactivity was found in the invasive fronts ([Supplementary material 3](#)).

Co-culture of normal PDL fibroblasts with SCC-25 cells, changes in gene expression

Normal human periodontal filament fibroblasts (PDLs), and SCC-25 cells were co-cultured for 7 days. After this time gene expression changes in PDLs and in SCC-25 cells were investigated, and compared with control cells. The controls received the same culture conditions, but they were not co-cultured with the partners. By this analysis, in the case of fibroblasts, changes of stroma-derived factor-1 (SDF-1) was investigated in co-culture vs. controls. 1.6-times significant ($p < 0.01$ by one-way ANOVA) reproducible increase of SDF expression was found in co-cultured fibroblasts compared with control PDL fibroblasts, which indicates the development of carcinoma-associated fibroblasts of PDLs.² In the same co-culture system gene expression changes of key genes for EMT were investigated in co-cultured SCC-25 cells vs. control SCC-25 cells. A significant increase of vimentin mRNA expression was detected ([Fig. 1A](#), [Supplementary material 4](#)), while E-cadherin mRNA expression was dramatically decreased ([Fig. 1B](#), [Supplementary material 4](#)) ($p < 0.05$). Snail was detected in control and in co-cultured SCC-25 cells; its gene expression increase in co-culture was not statistically significant ($p = 0.27$) ([Fig. 1C](#), [Supplementary material 4](#)). Significant ($p < 0.01$) correlation was found between gene expression of vimentin and snail (correlation coefficient: 0.95). Significant ($p < 0.01$) negative correlation was found

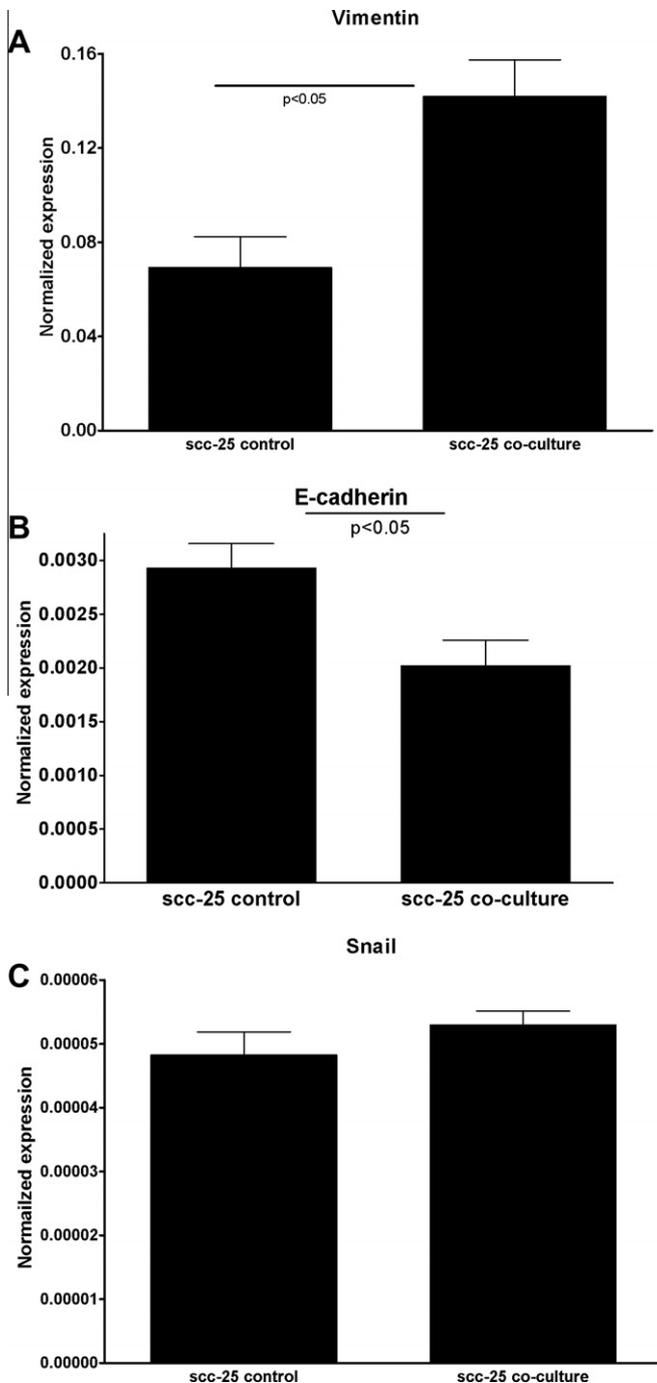


Figure 1 EMT-related gene expression changes in co-cultured SCC-25 cells. Key genes for EMT were investigated in co-cultured SCC-25 cells vs. control SCC-25 cells. A significant increase of vimentin mRNA expression was detected (A), while E-cadherin mRNA expression was dramatically decreased (B). Snail was detected in control and in co-cultured SCC-25 cells; its gene expression increase in co-culture was not statistically significant (C).

between gene expression of vimentin and E-cadherin (correlation coefficient: -0.81), and statistically significant ($p = 0.05$) correlation was found between gene expression of snail and E-cadherin (correlation coefficient: 0.37). These changes represent the characteristic gene expression changes involved in EMT.¹⁰

In fibroblasts E-cadherin-expression was not detected in control and in co-culture conditions. Vimentin and snail were high expressed, and did not change significantly in co-culture conditions ($p = 0.63$ and 0.51 , respectively).

These gene expression changes confirmed that, CAFs were produced from PDL fibroblasts and EMT occurred in SCC-25 cells during the 7-days-co-culture.

Regulation of the gene expression of TrkB in SCC-25 cells and of BDNF in fibroblasts

In the co-culture conditions gene expression changes of TrkB and of its main ligand BDNF were investigated both in PDLs and in SCC-25 cells. TrkB-gene-expression was not detected in PDLs in control and in co-culture conditions. Significant TrkB-gene-expression was detected in SCC-25 cells, confirming published data.^{9,10} Moreover, a significant and reproducible, in average 1.5-times increase of TrkB-gene-expression was measured in co-cultured SCC-25 cells compared to control culture (Fig. 2A, Supplementary material 4). In SCC-25 cells TrkB was also investigated at protein level, using a combination of immunoprecipitation and western blotting. In both control and co-cultured SCC-25 cells the full-length 145 kD (glycoprotein-145) band²⁰ and a lower band of truncated TrkB were detected,²² and the density of the 145 kD band was 1.3-times increased in co-culture compared to control cells (The density was determined by Image J software.). Non-nuclear extracts of TrkB-overexpressing-SH-SY5Y cells were used as positive controls,¹⁹ where the electrophoretic properties of the detected bands were identical (Fig. 2B). The specificity of the detected bands was tested by immunoprecipitation of the same samples with a blocking peptide-inactivated anti-TrkB antibody. The blocking peptide completely inhibited the detection of the bands (Supplementary material 5). In this regard, the expression of TrkB-glycoprotein could be stated in SCC-25 cells, and its upregulation in co-culture with fibroblasts was also proved at protein level.

Kupferman et al. described previously a detectable BDNF expression in HNSCC cell lines.¹⁰ Confirming these data, we also detected a low BDNF expression in SCC-25 cells, which did not change significantly in co-culture conditions ($p < 0.05$ by Kruskal–Wallis test). Interestingly, in control PDLs in average 12.32-times higher expression of BDNF was measured, which in co-culture conditions reproducibly, significantly further increased in average 3.23-times. These data indicate that fibroblasts, and in special, functional CAFs are a significant source for TrkB-ligands (Fig. 2C, Supplementary material 4). In further analysis we found that the gene expression of SDF is highly significantly ($p < 0.01$) correlated with the one of BDNF in (correlation coefficient: 0.785), meaning that the induction of BDNF in fibroblasts is an event closely related with induction of CAF-phenotype.

Inflammatory cytokines produced by SCC-25 cells in relation to BDNF-induction in fibroblasts

There are only scattered reports on the regulation of BDNF expression, and those studies were done in neurons,^{23,24} which revealed a potential involvement of inflammatory cytokines in the regulation of BDNF-expression. Based on this background the mRNA expression of TNF- α and IL-1 β was investigated in PDLs and in SCC-25 cells in control and in co-culture conditions.

IL-1 β was detected in both of SCC-25 cells and in PDLs, in control SCC-25 cells in average 86.73-times higher expression was detected. The high IL-1 β -gene expression remained in the SCC-25 cells also in co-culture. In PDLs: IL-1 β -gene expression significantly increased in average 2.7-times in co-culture conditions (Fig. 3A, Supplementary material 4).

Similarly, TNF- α was also detected in both of SCC-25 cells and in PDLs, in control SCC-25 cells in average 5.15-times higher expression was detected. The TNF- α -gene-expression 1.45-times significantly increased in SCC-25 cells in co-culture (Fig. 3B,

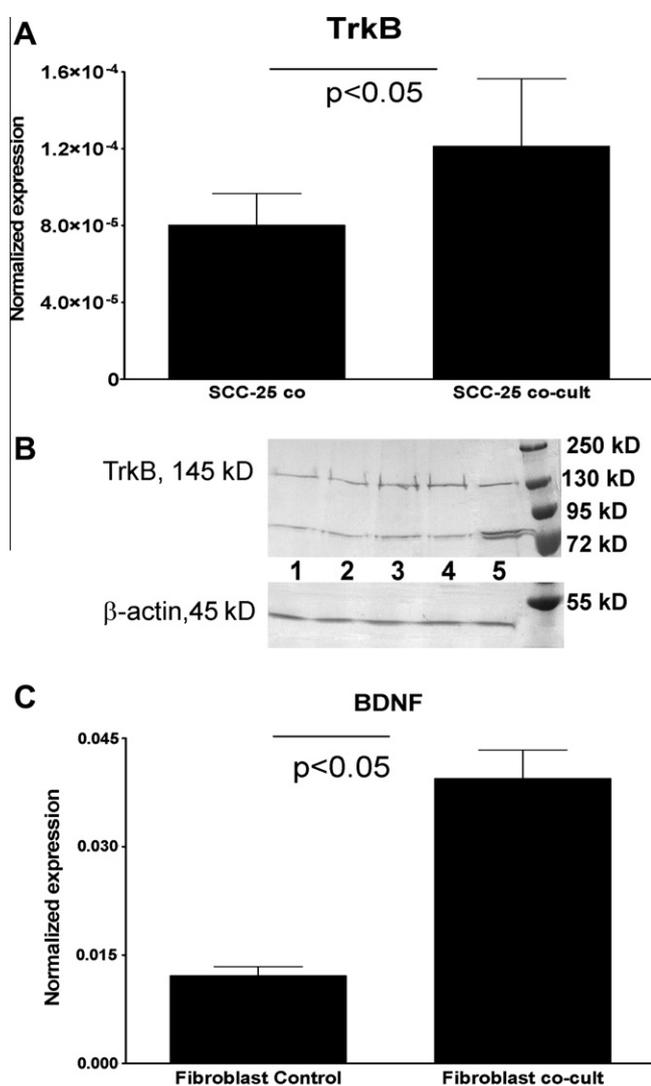


Figure 2 Analysis of TrkB and BDNF in co-cultures of PDL fibroblasts and SCC-25 cells. In the co-culture conditions gene expression changes of TrkB and BDNF were investigated in PDLs and SCC-25 cells. Significant TrkB-gene-expression was detected in SCC-25 cells, and it increased in co-culture condition at mRNA (A) and at protein level (B). In a combination of immunoprecipitation and western blot TrkB-specific bands were detected in SCC-25 cells in control and in co-culture conditions (B: protein extracts were loaded as follows: 1–2: control SCC-25 cells, 3–4: co-cultured SCC-25 cells, 5: TrkB-overexpressing SH-SY5Y cells; positive control), and showed an increase in co-culture. For controlling of the equal loading of protein samples β -actin western blot was done from the supernatants of TrkB-immunoprecipitated samples. A low BDNF expression was detected in SCC-25 cells (C), which did not change reproducibly in co-culture conditions. In control PDLs in average 12.32-times higher expression of BDNF was measured, which in co-culture conditions reproducibly, significantly further increased in average 3.24-times.

Supplementary material 4). BDNF gene expression showed a significant ($p < 0.05$) correlation with IL-1 β gene expression (correlation coefficient: 0.36).

Significant expression of inflammatory cytokines in co-culture conditions in PDLs and in SCC-25 cells and their co-culture-related increases raise the potential for these factors to regulate the BDNF-expression in co-cultured fibroblasts (CAFs).

Discussion

In recent reports a variable, but consistent expression of TrkB could be observed in HNSCC tissues and cell lines, whereas no significant expression was seen in the healthy control tissue. As a function, not the acceleration of cell proliferation, but a role in pre-

vention of anoikis of TrkB was suggested.^{25,26} Kupferman et al. in 2010 described a co-expression of TrkB and brain-derived neurotrophic factor (BDNF) in human HNSCC, and found functional proofs for the involvement of the BDNF-TrkB axis in the progression of HNSCC.¹⁰ Moreover, they described a direct association between TrkB function and EMT in HNSCC cell lines. Epithelial-mesenchymal transition (EMT) is implicated in the progression of primary tumors towards metastases. Increasing evidence suggests that EMT plays a specific role in the migration of cells from a primary tumor into the circulation and may provide a rationale for developing more effective cancer therapies.²⁷ At the same time, carcinoma cells seem to recruit normal fibroblasts into tumor masses and then force the conversion of these cells into special, carcinoma-associated fibroblasts in order to promote tumor growth and progression. CAFs release elevated levels of SDF-1, which is a functional characteristic of them.² Investigation of the fibroblast-to-CAFs conversion requires dynamic human in vitro models. In the current work we described such a co-culture model of PDL fibroblasts and SCC-25 oral squamous cell carcinoma cells, which results in conversion of CAFs from normal fibroblasts. In the same model EMT occurs in SCC-25 cells, representing its key-events: detection of snail-expression, increase of vimentin production and significant reduction of E-cadherin (Figs. 1 and 4). Furthermore, our study provides evidence that a major source of the main TrkB ligand: BDNF is the carcinoma-associated fibroblast (CAF). This finding describes a novel mechanism for the involvement of the BDNF-TrkB-axis in tumor progression (Fig. 4).

The co-culture system has the advantage against a conditioned medium system³ that simultaneous bi-directional changes can be analysed and statistically evaluated. This is the first clear demonstration, which shows that conversion of oral fibroblasts to CAFs and EMT in oral carcinoma cells are simultaneous, coordinated events. During this coordinated set of events: BDNF was induced in CAFs, which were converted from PDL fibroblasts, and simultaneously TrkB receptor expression was increased in oral carcinoma cells (Fig. 4).

The role of the inflammatory cytokines in induction of BDNF-expression was described in neurons.^{23,24} This regulatory pathway is also plausible in CAFs (Figs. 3 and 4), the source of those cytokines could be the SCC-25 cells, or more importantly the EMT-SCC-25 cells, but also the fibroblasts themselves (Fig. 3).

In addition, TNF- α represents also a potential factor for regulating the induction of CAFs and initiation of EMT in tumor cells. TNF- α -expression was also significant in both oral fibroblasts and in carcinoma cells; moreover, in EMT-carcinoma cells its expression significantly increased (Fig. 3B). A role of TNF- α in regulating EMT has been reported in several recent reports.^{28,29} Furthermore, our results confirmed the previous conclusions of Koontongkaew and colleagues that the interactions between cancer cells, the extracellular matrix and fibroblasts, are mediated by cytokines and chemokines, and play important roles in the progression of HNSCC.³⁰

The novel findings described in this study initiate a more detailed mechanistic analysis of the regulatory pathways of TNF- α , IL-1 β and other factors on the CAF-induction and EMT, which will be the issue of a following report.

Conflict of interest statement

None declared.

Authors contribution

J. Dudas, C. Falkeis, G.M. Sprinzl and H. Riechelmann participated in the design of the study, M. Bitshe and J. Dudas performed cell culture, western blot, gene expression, and immunocytochem-

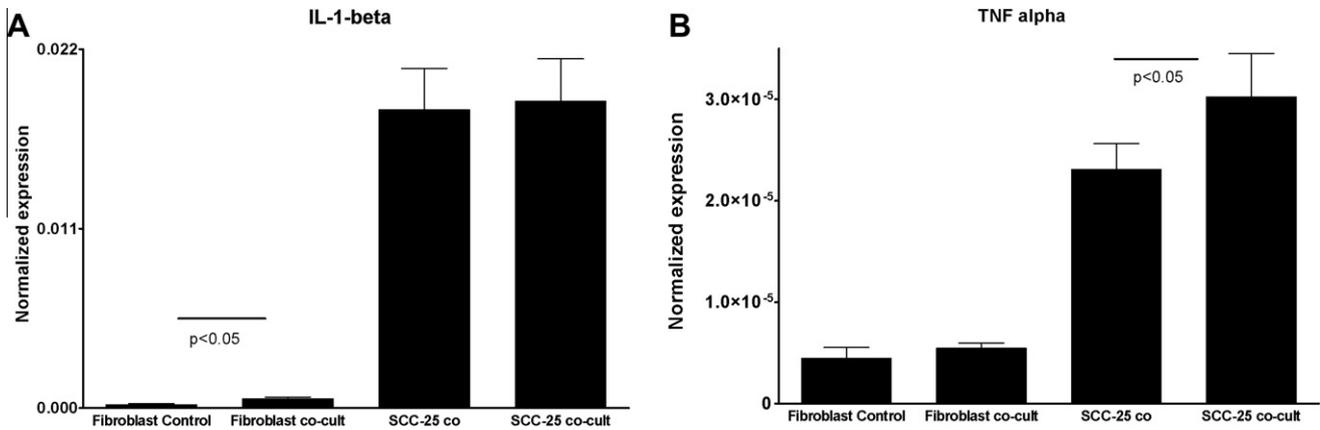


Figure 3 Gene expression of IL-1 β (A) and TNF- α (B) in PDL fibroblasts and SCC-25 cells in control and co-culture conditions. IL-1 β was detected in both of SCC-25 cells and in PDLs, in control SCC-25 cells in average 86.73-times higher expression was detected. The high IL-1 β -gene expression remained in the SCC-25 cells also in co-culture. In PDLs: IL-1 β -gene expression increased in average 2.7-times in co-culture conditions (A). Similarly, TNF- α was also detected in both of SCC-25 cells and in PDLs, in control SCC-25 cells in average 5.15-times higher expression was detected. The TNF- α -gene-expression increased significantly 1.45-times in SCC-25 cells in co-culture (B).

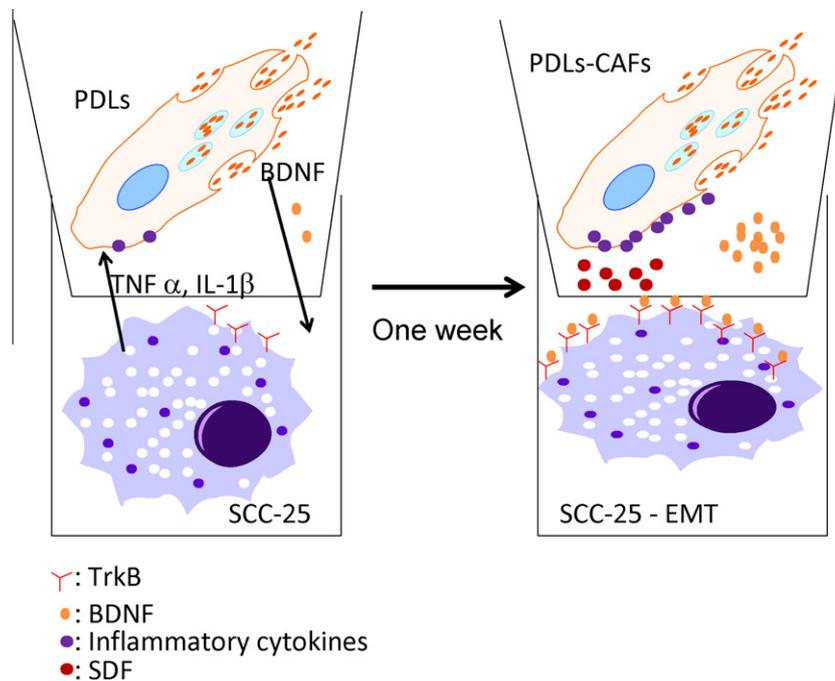


Figure 4 Suggested mechanism for the involvement of the BDNF-TrkB-axis in the coordination of CAF-induction and EMT. SCC-25 cells express sustained levels of TrkB and inflammatory cytokines. Normal fibroblasts express BDNF, which is the main ligand of TrkB. Inflammatory cytokines released from SCC-25 cells might contribute to the upregulation of BDNF in PDL fibroblasts. At the same time these cytokines are involved in the induction of CAFs from fibroblasts. CAFs release increased levels of BDNF, which is related with increased TrkB-expression in SCC-25 cells. In co-culture SCC-25 suffer mesenchymal transition, partly due to the TrkB receptor signaling. For the preparation of this figure a toolkit of Motifolio (Ellicott City, MD, USA) was used.

ical experiments, C. Falkeis, V. Schartinger and M. Bitsche prepared, performed and analyzed immunohistochemical stainings, J. Dudás, V. Schartinger, and H. Riechelmann have done statistical analysis. J. Dudás have drafted the manuscript. G. Sprinzl, H. Riechelmann and V. Schartinger revised the manuscript critically for important intellectual content; and have given final approval of the version to be published. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.oraloncology.2010.11.002.

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